NUCLEIC-ACID THERAPEUTICS: BASIC PRINCIPLES AND RECENT APPLICATIONS

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The sequencing of the human genome and the elucidation of many molecular pathways that are important in disease have provided unprecedented opportunities for the development of new therapeutics. The types of molecule in development are increasingly varied, and include antisense oligonucleotides and ribozymes. Antisense technology and catalytic nucleic-acid enzymes are important tools for blocking the expression of abnormal genes. One FDA-approved antisense drug is already in the clinic for the treatment of cytomegalovirus retinitis, and other nucleic-acid therapies are undergoing clinical trials. This article reviews different strategies for modulating gene expression, and discusses the successes and problems that are associated with this type of therapy.

EXOGENOUS NUCLEIC ACIDS
In this context, synthetic oligonucleotides of varying chemistry (typically 16–25 nucleotides), which are introduced into cells by various means, or simply (although inefficiently) by concentration-driven endocytosis.

ANTISENSE
Reverse complement of any DNA or RNA sequence.

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With their promise of high specificity and low toxicity, many believe that gene-targeted therapies will lead to a revolution in cancer therapeutics[1]. Numerous gene-therapy strategies are under development, some of which use nucleic-acid-based molecules to inhibit gene expression at either the transcriptional or post-transcriptional level[2]. This strategy clearly has other potential applications, including in cardiovascular[3,4], inflammatory[5,6] and infectious diseases[7–10], as well as organ transplantation[11].

Although conceptually elegant, the prospect of using nucleic-acid molecules for treating human malignancies and other diseases remains tantalizing, but uncertain[12]. The main cause of this uncertainty is the apparent randomness with which these materials modulate the expression of their intended targets. It is a widely held view that molecule delivery, and selection of which messenger RNA sequence to physically target, are core stumbling blocks that hold up progress in the field. In this review, we recapitulate the development of nucleic-acid drugs for modulating gene expression, discuss newer strategies for solving the problems alluded to above, and detail attempts at using these molecules therapeutically. In so doing, we hope to both educate the reader who is unfamiliar with this literature, and convince those who are sceptical that this remains a viable approach to 'on demand' manipulation of gene expression.

Modulating gene expression

The notion that gene expression could be modified through the use of EXOGENOUS NUCLEIC ACIDS derives from studies by Paterson *et al.*[13], who first used single-stranded DNA to inhibit translation of a complementary RNA in a cell-free system in 1977. The following year, Zamecnik and Stephenson[14] showed that a short (13-mer) DNA oligodeoxynucleotide that was ANTISENSE to the Rous sarcoma virus could inhibit viral replication in culture. On the basis of this work, Zamecnik and Stephenson are widely credited for having first suggested the therapeutic utility of antisense nucleic acids. In the mid 1980s, the existence of naturally occurring antisense RNAs and their role in regulating gene expression was shown[15,16]. These observations were particularly important, because they lent credibility to the belief that 'antisense' was more than just a laboratory phenomenon, and encouraged belief in the hypothesis that reverse-complementary antisense nucleic acids could be used in living cells to manipulate gene expression. These seminal papers, and the thousands that have followed, have stimulated the development of technologies that use nucleic acids to manipulate gene expression. As will be discussed below, virtually all of the available methods rely on some type of nucleotide-sequence recognition for targeting

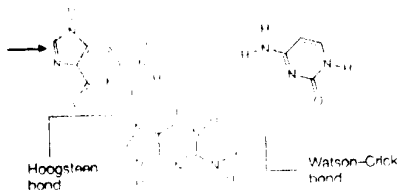

Figure 1 | **Triple-helix formation at the nucleotide level.** Shows the formation of Watson–Crick (red) and Hoogsteen bonds (black) between duplex pairs and the third strand (the arrow points to a single base of the third strand). Blue, guanine residue (purine); pink, cytosine residue (pyrimidine).

TRIPLE HELIX FORMING OLIGODEOXYNUCLEOTIDES (TFOs) A synthetic, single-stranded oligodeoxynucleotide which, through Hoogsteen-bond formation, hybridizes to purine/pyrimidine-rich sequences in double-stranded DNA. Formation of stable triple helices can prevent the unwinding that is necessary for transcription of the targeted region or block the binding of transcription-factor complexes.

MAJOR GROOVE AND MINOR GROOVE Channels formed by the twisting of two complementary DNA strands around each other to form a double helix. The major groove is ~22 Å wide and the minor groove is ~12 Å wide.

HOOGSTEEN BOND Triple-helix-forming oligonucleotides hybridize with purine bases that comprise polypurine/polypyrimidine tracks in the DNA. The hydrogen bonds that are formed under these conditions are referred to as Hoogsteen bonds after the individual who first described them. They can form in parallel or antiparallel (reverse-Hoogsteen) orientations.

NUCLEOSOME A packing unit for DNA within the cell nucleus, which gives the chromatin a 'beads-on-a-string' structure. The 'beads' consist of complexes of nuclear proteins (histones) and DNA, and the 'string' consists of DNA only. A histone octamer forms a core around which the double-stranded DNA helix is wound twice.

LEXITROPSIN A molecule that stereogenetically reads the base sequence of double-stranded DNA.

RIBOZYME RNA molecule that contains one of a variety of catalytic motifs that cleave RNA to which it hybridizes.

DNAzyme A DNA molecule that contains a catalytic motif that cleaves RNA to which it hybridizes.

specificity, but differ as to where and how they perturb the flow of genetic information.

Strategies for modulating gene expression can be thought of as being either 'anti-gene' or anti-mRNA (see below; reviewed in REF. 2). Anti-gene strategies focus primarily on gene targeting by homologous recombination[17,18], or by TRIPLE HELIX FORMING OLIGODEOXYNUCLEOTIDES (TFOs)[19]. As homologous recombination involves vector technology and — at least at the present time — is much too inefficient for clinical use, it will not be considered further in this discussion. TFOs bind in the MAJOR GROOVE of duplex DNA in a sequence-specific manner[20]. Gene targeting with these molecules is constrained by the fact that TFOs require runs of purines on one strand and pyrimidines on the other (~10–30 nucleotides (nts) in length) for stable hybridization. The TFO can be composed of either polypurine or polypyrimidine tracts, but hybridization always occurs on the purine strand of the duplex through the formation of HOOGSTEEN BONDS (FIG. 1).

Successful use of this strategy for blocking transcription and inducing specific mutations, both *in vitro* and *in vivo*, has been reported (reviewed in REF. 20). Although the frequency of such events is typically <1%, Glazer and co-workers[21] have reported a system in which desired mutations can be induced in ~50% of cells, indicating that genuine clinical utility might be possible. This general approach has also been used for inducing mutations that can actually repair a gene that has been made defective by inherited or acquired point mutation. Work to support this concept using chimeric DNA–RNA oligonucleotides has also been reported, but again, the frequency of such repairs, in most cases, has been far too low to be of clinical use at this time[22].

Short, double stranded (ds)DNA decoy molecules have also been used to disrupt gene expression at the level of transcription[23]. These oligodeoxynucleotides are designed to compete for transcription-factor complexes, with the ultimate goal of attracting them away from the promoter that they would ordinarily activate. For many technical reasons, including limited gene accessibility in the NUCLEOSOME structure, the clinical application of these methods has not progressed at a rapid rate. An alternative approach, using polyamides, or LEXITROPSINS, has been described by Dervan and colleagues[24–26]. These small molecules have the ability to diffuse into the nucleus, where they can contact dsDNA in the minor groove, thereby impeding transcription by preventing unwinding of the duplex, or by preventing the binding of transcription-factor complexes to the gene promoter. DNA accessibility, and maintaining the appropriate 'register' of the polyamides for the desired sequence recognition, are problems with this method that remain to be solved[27].

A larger body of work has focused on destabilizing mRNA. This approach, although less favourable than anti-gene strategies from a stoichiometric point of view, is nonetheless attractive, because mRNA, unlike the DNA of a given gene, is — theoretically— accessible to attack while being transcribed, transported from the nucleus or translated. Two nucleic-acid-based strategies have emerged for blocking translation. One strategy uses oligoribonucleotides. Similar to the strategy of the DNA decoys, the RNA decoys are designed to provide alternate, competing binding sites for proteins that act as translational activators or mRNA-stabilizing elements[28,29]. By attracting away the desired protein, the decoy can prevent translation, or induce instability and, ultimately, destruction of the mRNA. Recent studies on human α-globin mRNA are of interest in this regard. Stability determinants for this mRNA species have been defined in sufficient detail so that it can be used as a model system for testing the hypothesis that altering mRNA stability with decoys will be a useful form of therapy[29–31].

The other strategy for destabilizing mRNA is the more widely applied antisense strategy, which uses RIBOZYMES, DNAzymes, antisense RNA or antisense DNA (ODN). The antisense approach to modulating gene expression has been the subject of numerous authoritative reviews, and will not be discussed in great detail here[2,32–34]. Simply stated, delivering a reverse-complementary — that is, 'antisense' — nucleic acid into a cell in which the gene of interest is expressed should lead to hybridization between the antisense sequence and the mRNA of the targeted gene. Stable mRNA–antisense duplexes can interfere with the splicing of heteronuclear RNA into mature mRNA[34,35], block translation of completed message[36,37] and — depending on the chemical composition of the antisense molecule —lead to the destruction of the mRNA by binding of endogenous nucleases, such as RNaseH[38,39], or by intrinsic enzymatic activity engineered into the sequence, as is the case with ribozymes[40,41] and DNAzymes[42,43] (FIG. 2).

Nucleic acids with catalytic activity

Ribozymes and DNAzymes bind to substrate RNA through Watson–Crick base pairing, which offers sequence-specific cleavage of transcripts. At least six classes of ribozyme have been described. Two ribozymes, the 'hammerhead' ribozyme and the 'hairpin' ribozyme, have been extensively studied owing to their small size and rapid kinetics[44,45]. The catalytic motif is surrounded by flanking sequence that is responsible for 'guiding' the ribozyme to its mRNA target and giving stability to the structure. With the hammerhead ribozyme, cleavage is dependent on divalent cations, such as magnesium, and can occur after any NUH

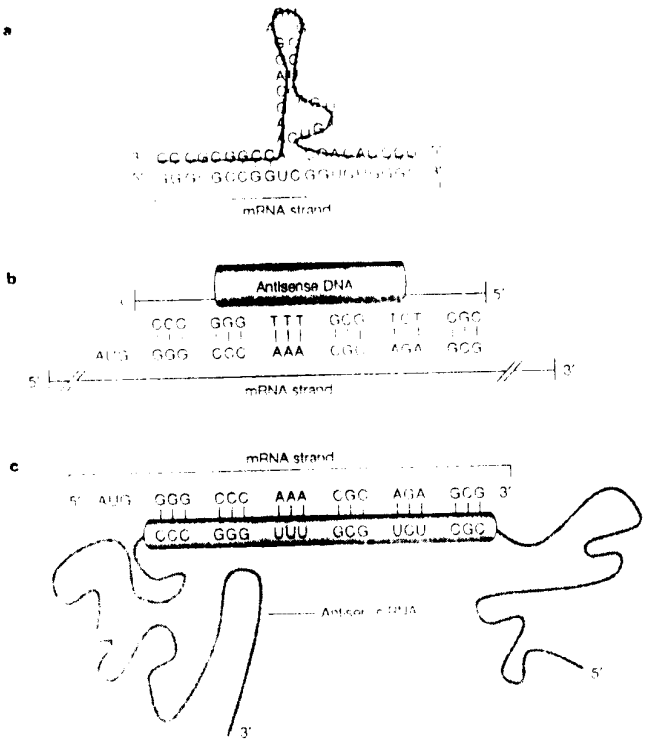

Figure 2 | **Strategies for inhibiting translation.** Diagrammatic representations of **a** | a hammerhead ribozyme (DNAzymes have similar RNA-cleaving capabilities, but the catalytic motif is composed of DNA nucleotides, hence the name), **b** | an antisense oligodeoxynucleotide, and **c** | antisense RNA. Note that targeting specificity is conveyed in each case by Watson–Crick base pairing between complementary sequences. From REF. 2 © (1998) American Society of Hematology, used by permission. mRNA, messenger RNA.

triplet within the target RNA sequence, for which 'N' represents any nucleotide, 'U' represents uracil and 'H' represents adenine, cytosine or uracil[46,47]. If ribozymes are to work effectively as 'enzymes,' they must not only bind substrate RNA but also dissociate from the cleavage product to act on further substrates. Dissociation from the cleavage product might, in fact, be an important rate-limiting step that controls their usefulness[48,49]. Consideration of reaction kinetics indicates that ribozymes might have a theoretical advantage over RNase-H-dependent antisense oligonucleotides, but to the best of our knowledge, this has not been shown consistently *in vivo*. Ribozymes can be expressed from a vector that offers the advantage of continued production of these molecules intracellularly[50,51], a property that — at least until recently — was not possible with antisense DNA[52]. However, it is well known that stable transduction of primary cells *in vivo* has substantial technical problems, which will not be discussed further. Progress has been made recently in synthesizing stable forms of these molecules, so that they might be delivered directly to cells both *in vitro* and *in vivo*[53].

DNAzymes have evolved from the seminal work of Breaker and Joyce[54], who first showed that DNA, as well as RNA molecules, could act enzymatically and cleave a nucleic-acid substrate. Similar to ribozymes, DNAzymes have a catalytic domain that is flanked by two substrate-recognition domains. After binding to their RNA substrate, DNAzymes can cleave sequences that contain purine–pyrimidine junctions. DNAzymes have some theoretical advantages over ribozymes. DNA is more stable than RNA, it is easier to synthesize, and the turnover rates for some of the DNAzymes are reported to be higher than some ribozymes[42]. Nevertheless, constant improvements in both DNAzyme[55] and ribozyme chemistry make this a 'moving target' in terms of which chemistry is better[56]. Although experience with DNAzymes as potential therapeutic agents is limited[41], these molecules might prove worthy in the clinical setting.

RNA interference

A newly developing approach for targeting mRNA is called post-transcriptional gene silencing, or RNA interference (RNAi)[57–58] (FIG. 3). RNAi is the process by which dsRNA targets mRNA for destruction in a sequence-dependent manner. The mechanism of RNAi initially involves processing of long (~500–1,000 nucleotides) dsRNA into 21–25 base-pair (bp) 'trigger' fragments[59] by a member of the RNase-III family of nucleases called DICER[60–62]. When incorporated into a larger, multicomponent nuclease complex named RISC (RNA-induced silencing complex), the processed trigger strands form a 'guide sequence' that targets the RISC to the desired mRNA sequence and promotes its destruction[63]. RNAi has been used successfully for gene silencing in various experimental systems, including petunias, tobacco plants, neurospora, *Caenorhabditis elegans*, insects, planaria, hydra and zebrafish. The use of long dsRNA to silence expression in mammalian cells has been tried, largely without success[64]. More recent reports using short interfering RNA (siRNA; see below) seem to be more promising[64]. It has been suggested that mature, as opposed to embryonic, mammalian cells recognize these long dsRNA sequences as invading pathogens. This triggers a complex host-defence reaction that effectively shuts down all protein synthesis in the cell through an interferon-inducible serine/threonine-kinase enzyme called protein kinase R (PKR). PKR phosphorylates the α-subunit of eukaryotic initiation factor-2 (EIF-2α), which globally inhibits mRNA translation. The long dsRNA also activates 2′,5′-oligoadenylate synthetase, which in turn activates RNase L. RNase L indiscriminately cleaves mRNA. Cell death is the understandable result of these processes. Recently, a number of reports have suggested that siRNA strands — RNA double strands of ~21–22 nucleotides in length — do not trigger this host-defence response, and therefore might be able to silence expression in mammalian somatic cells if appropriately modified to contain 3′-hydroxy and 5′-phosphate groups[65–68]. The universality of this approach, and the types of gene that can be modified using this strategy in mammalian cells, remain unknown at this time.

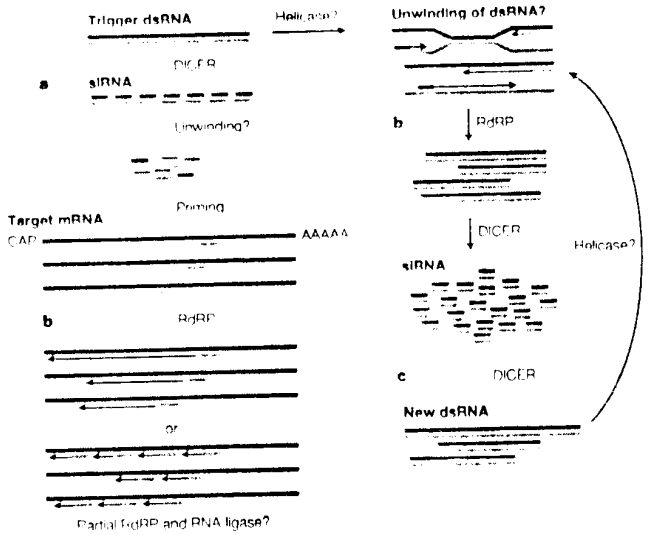

Figure 3 | **Hypothetical RNAi mechanism. a** | In the 'initiation' stage of RNA interference (RNAi), a small amount of trigger double-stranded (ds)RNA is processed into short interfering (si)RNA by an enzyme called DICER (light blue arrow), which is used as an RdRP primer. **b** | The RdRP reaction converts target messenger RNAs into new dsRNAs (next generation of trigger dsRNAs), which are then processed into new siRNAs, establishing a self-sustaining cycle of RNAi 'maintenance' (green arrows). **c** | Replication of 'trigger' or newly synthesized dsRNA by RdRP would amplify the potency of RNAi by further increasing the amount of siRNA, as both sense and antisense strands of trigger dsRNA and siRNA could then be used. However, the *in vivo* significance of this pathway (dark grey arrows) has not yet been established. It also remains unclear if the 'amplification' steps take place in mammalian cells. RdRP, RNA-dependent RNA polymerase; helicase, unwinding enzyme. Redrawn from REF. 57 © (2001), with permission from Elsevier Science.

Altering RNA splicing

Finally, the strategy of manipulating gene expression by altering RNA processing, as opposed to by mRNA destruction, is also worth mentioning, as significant progress seems to have been made in this area. Kole and colleagues developed this approach using a model system based on human thalassaemia[69,70]. Thalassaemias are highly prevalent human blood disorders that are characterized by faulty haemoglobin production and concomitant red-cell destruction that results in anaemia. The genetic mutations that are responsible for these diseases are well characterized, and often involve aberrant splicing. Kole's group showed that treatment of mammalian cells that were stably expressing a human β-globin gene with antisense oligonucleotides that were targeted at the aberrant splice sites blocked the abnormal splicing, thereby allowing the normal splice site to be used. Correction of splicing was oligo-dose dependent and, importantly, led to accumulation of normal human β-globin mRNA and polypeptide in cells[69]. More recently, correction has been accomplished in blood cells derived from thalassaemic patients[71]. This result would clearly have important clinical consequences if such treatment could be made effective at the level of the haematopoietic stem cell. These same workers suggest that this approach might also be useful in the treatment of cancer[71].

Increasing oligonucleotide stability

Initial work with antisense DNA was carried out with unmodified, natural molecules. It soon became clear, however, that native DNA was subject to relatively rapid degradation, primarily through the action of 3′ exonucleases, but also as a result of endonuclease attack. Molecules destined for the clinic, and those used for experimental purposes, are now routinely modified to enhance their stability, as well as the strength of their hybridization with RNA (see REFS 73,74 for further details). Oligonucleotide drugs need to meet certain physical requirements to make them useful. First, they must be able to cross cell membranes and then hybridize with their intended target. The ability of an ODN to form a stable hybrid is a function of its binding affinity and sequence specificity. Binding affinity is a function of the number of hydrogen bonds that are formed between the ODN and the sequence to which it is targeted. This is measured objectively by determining the temperature at which 50% of the double-stranded material is dissociated into single strands, which is known as the melting temperature, or T_m. mRNA-associated proteins and tertiary structure also govern the ability of an ODN to hybridize with its target by physically blocking access to the region that is being targeted by the ODN. Finally, it is also clear that ODNs should exert little in the way of non-sequence-related toxicity[75], and should remain stable in the extracellular and intracellular milieu in which they are situated. Meeting all these requirements in any one molecule has turned out to be a demanding task. Satisfying one criterion is often accomplished at the expense of another. It is also worth noting that the more complex the molecule, the more expensive is its synthesis. In an age of increasing cost consciousness, this too becomes an important design consideration.

First-generation antisense molecules were designed to make the internucleotide linkages — the backbone on which the nucleosides are hung — more resistant to nuclease attack. This was accomplished primarily by replacing one of the non-bridging oxygen atoms in the phosphate group with either a sulphur or a methyl group. The former modification, which is called a phosphorothioate oligodeoxynucleotide, proved highly successful, because these molecules are relatively nuclease resistant, they are charged and therefore water soluble, and they activate RNase H. All of these properties are desirable, and virtually all of the clinical trials done so far have been carried out with this chemistry, although trials using so-called 'second-generation molecules' (mixed backbone/chimeric oligonucleotides) will shortly begin. Second-generation molecules were developed to overcome the disadvantageous properties of the phosphorothioates. A primary strategy that was used was to remove the phosphorothioate linkages to the greatest extent possible. This was often done by flanking a phosphorothioate core with nuclease-resistant nucleosides — often with 2′-*O* sugar modifications — that rendered the molecules more RNA like, and therefore gave tighter binding to the target.

Many chemical modifications to the phosphodiester linkage have been made. Two of the more interesting modifications that are now under development are peptide nucleic acids (PNAs)[77] and morpholino oligodeoxynucleotides (PMOs)[78]. These compounds are essentially nuclease resistant. PNAs represent a more radical approach to the nuclease resistance problem, as the phosphodiester linkage is completely replaced with a polyamide (peptide) backbone. They both form extremely tight bonds with their RNA targets and probably exert their effects by blocking translation, as neither molecule effectively activates RNase H. Whether it is necessary to preserve the ability of these molecules to activate RNase H is controversial[37], but many workers in the field still believe that molecules with this capability are likely to be more effective, at least in the clinical setting. As these molecules do not move freely across cell membranes, they must be injected or transfected into cells. Finally, PNAs are also sensitive to local ionic concentration and do not hybridize as well under physiological conditions.

Nucleic-acid drugs in the clinic

Diseases that are characterized by overexpression or inappropriate expression of specific genes, or genes that are expressed by invading microorganisms, are candidates for gene-silencing therapies. For this reason, the earliest clinical trials with these agents have been against human immunodeficiency virus (HIV)77–79 and patients with cancer80. Malignant diseases, in particular, are attractive candidates for this therapeutic approach, if for no other reason than that conventional cancer therapies are highly toxic. As antisense strategies are directed against genes that are aberrantly expressed in diseased cells, it might reasonably be expected that this approach will engender fewer and less serious side effects, as normal cells should not be affected. There were concerns that this might not be the case when preclinical studies on primates with phosphorothioate compounds resulted in the death of some animals. However, investigation of these occurrences showed that they took place after rapid bolus intravenous infusions at concentrations exceeding 5–10 μg ml^{-1}, and that they were probably due to complement activation and vascular collapse[81].

MORPHOLINO OLIGODEOXYNUCLEOTIDE (PMO). The base is attached to a morpholino instead of a ribofuranosyl ring, and the backbone is composed of a phosphorodiamidate linkage.

Box 1 | First approved nucleic-acid drug

Vitravene (sodium fomivirsen), an antiviral drug that was developed by ISIS Pharmaceuticals and is marketed by CIBAVision, was approved by European and US regulatory authorities in July 1999 and August 1998, respectively. Vitravene is used to treat an inflammatory viral infection of the eye (retinitis) that is caused by the cytomegalovirus (CMV). CMV often infects immunocompromised patients, and patients with uncontrolled AIDS are particularly at risk. One or both eyes can be affected, and it is not unusual for patients to suffer severe visual impairment or blindness as a result of untreated infections. Treatment of CMV retinitis is problematic, in particular for patients who cannot take, do not respond or become resistant to standard antiviral treatments for CMV infections, such as ganciclovir, foscarnet and cidofovir[74]. Vitravene is an antisense phosphorothioate 21-mer oligonucleotide has a sequence that is complementary to messenger RNA that is transcribed from the main immediate–early transcriptional unit of CMV[75,76].

This experience was therefore a useful reminder that, in addition to side effects resulting from the suppression of the targeted gene, side effects related to the chemical backbone of the oligonucleotide should also be anticipated. In the case of phosphorothioates, this problem was easily addressed by infusing material continuously, or slowly, and at lower doses. In actual use in the clinic, phosphorothioates have proved to be remarkably well tolerated (BOX 1). Abnormalities related to the backbone include transient fever, fatigue, nausea and vomiting, mild to moderate thrombocytopaenia and transient prolongation of partial thromboplastin time (PTT; 1.25–1.75 ×), which is fortunately unassociated with any signs of overt clinical bleeding[82–85]. At present, several clinical studies have been carried out using a number of different oligonucleotides. Below, we review some of the more recent clinical studies that have been carried out on patients with malignant, inflammatory, cardiac and infectious diseases (summarized in TABLE 1).

Targeting apoptosis inhibitors in oncology

BCL2: cancer treatment. Targeting B-cell lymphoma protein 2 (BCL2) is a promising example of triggering apoptosis in tumour cells. BCL2 is an important regulator of programmed cell death, and its overexpression has been implicated in the pathogenesis of some lymphomas[86]. Resistance to chemotherapy, at least *in vitro*, might also be related to *BCL2* overexpression[87,88]. Laboratory studies have shown convincingly that exposing cells to an oligonucleotide targeted to *BCL2* will specifically decrease the amount of targeted mRNA and protein (six–eightfold reduction). For all of these reasons, there is a great deal of interest in targeting *BCL2* for therapeutic purposes[89]. Several clinical trials with a *BCL2*-targeted antisense molecule have been reported, both alone[81,90] and with supplementary chemotherapy[84,91,92]. Studies with the oligonucleotide alone have not shown consistent, strong antitumour responses. The addition of chemotherapy might be helpful in this regard. An issue with several of these studies is lack of correlation of tumour responses with significant effects on BCL2 protein expression. The mechanism of action of the compound is not entirely clear.

Transcription-factor targeting in oncology

c-MYB: bone-marrow purging. The normal homologue of the avian myeloblastosis virus oncogene (*v-myb*) is a proto-oncogene called *c-MYB*. c-*MYB* encodes a protein (MYB), which is a regulator of cell-cycle transition and cellular maturation, primarily in haematopoietic cells, but in other cell types as well. A recently published study was designed to test the hypothesis that an effectively delivered, appropriately targeted ODN might provide a proof of concept about the ability to target a specific mRNA and thereby kill tumour cells selectively[93]. To test this hypothesis, an ODN targeted to the c-*MYB* proto-oncogene was used to purge marrow autografts that were administered to patients with allograft-ineligible chronic myelogenous leukaemia (CML). $CD34^+$ marrow cells were purged

with ODN for either 24 ($n = 19$) or 72 ($n = 5$) hours (FIG. 4). Post-purging, *c-MYB* mRNA levels declined substantially in ~50% of patients. Analysis of *BCR–ABL* (breakpoint cluster region–Abelson murine leukaemia viral oncogene homologue) expression in a surrogate stem-cell assay indicated that purging had been accomplished at a primitive cell level in >50% of patients. Cytogenetics were evaluated at day 100 in surviving patients who did not require administration of unpurged 'rescue' marrow for engraftment ($n = 14$). (All purging protocols require storage of untreated marrow as a 'back-up', in case the purged material does not engraft.) Whereas all patients were ~100% Ph^+ (Philadelphia chromosome positive) pre-transplant, two patients had complete cytogenetic remissions, three patients had <33% Ph^+ metaphases and eight remained 100% Ph^+. The marrow of one patient yielded no metaphases, but fluorescence *in situ* hybridization (FISH) evaluation ~18 months post-transplant revealed that ~45% of cells were *BCR–ABL*$^+$, indicating that six

Table 1 | **Summary of recently published clinical trials with nucleic-acid drugs**

Target	Type of study	No. of patients	Diagnosis	Dose range	Treatment duration	Administration	Remissions	Refs
ICAM-1	Multicentre; placebo controlled; double blind	75	Crohn's disease	0.5 mg	2 days–4 weeks	SC	Not significant	106
	Placebo controlled; double blind	20	Crohn's disease	0.5–2 mg kg^{-1}	26 days	2 hours IV infusion	47% steroid-free remissions	6
PKC-α	Phase I	36	Advanced cancer	0.15–6 mg kg^{-1} d^{-1}	3 days per week for 3 weeks every 4 weeks	2 hours IV infusion	2 CR	82
	Phase I	21	Advanced cancer	0.5–3 mg kg^{-1} d^{-1}	21 days every 4 weeks	Continuous IV infusion	3 responses	85
BCL2	Phase I	21	Relapsed NHL	4.6–196.8 mg m^{-2} d^{-1}	14 days	Continuous SC infusion	1 CR, 2 minor responses	83
BCL2 combined with dacarbazine	Phase I/II	14	Advanced malignant melanoma	0.6–6.5 mg kg d^{-1}	14 days every 4 weeks	Continuous IV infusion	1 CR, 2 PR 3 minor responses	91
BCL2 combined with mitoxantrone	Phase I/II	26	Metastatic prostate cancer	0.6–5 mg kg^{-1} d^{-1}	14 days every 28 days	Continuous IV infusion	2 decreases in PSA	84
Fomivirsen CMV	Multicentre; randomized; prospective	29	CMV retinitis in AIDS patients	165 μg	Once per week	Intravitreously	Time to progression 71 versus 13 days	147
h-RAS	Phase I	23	Advanced cancer	0.5–10 mg kg^{-1} d^{-1}	14 days every 3 weeks	Continuous IV infusion	4 stable	96
c-RAF kinase	Phase I	34	Advanced cancer	1–5 mg kg^{-1} d^{-1}	21 days every 4 weeks	Continuous IV infusion	2 stable diseases	119
	Multicentre Phase II	22	SCLC and NSCLC	2 mg kg^{-1} d^{-1}	21 days every 4 weeks	Continuous IV infusion	No responses	148
	Phase I	22	Advanced cancer	6–30 mg kg^{-1} d^{-1}	Weekly	24 hours IV infusion	No responses	99
c-MYC	Multicentre; placebo controlled	78	After PTCA	1–24 mg d^{-1}	Single dose	Intracoronary	No responses	108
	Placebo controlled	85	After coronary stent implantation	10 mg d^{-1}	Single dose	Intracoronary	No responses	109
	Pilot study	12	Malignant astrocytoma	2 mg 10^{-1} cells	6 hours	*Ex vivo*	2 CR 6 PR	118

AS, antisense; BCL2, B-cell lymphoma protein 2; CMV, cytomegalovirus; CR, complete remission; ICAM-1, intercellular adhesion molecule-1; IGF1R, insulin-like-growth-factor-1 receptor; IV, intravenous; c-MYC, myelocytomatosis viral oncogene homologue; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PKC-α, protein kinase C α; PR, partial remission; PSA, prostate-specific antigen; PTCA, percutaneous transluminal coronary angioplasty; SC, subcutaneous; SCLC, small-cell lung cancer.

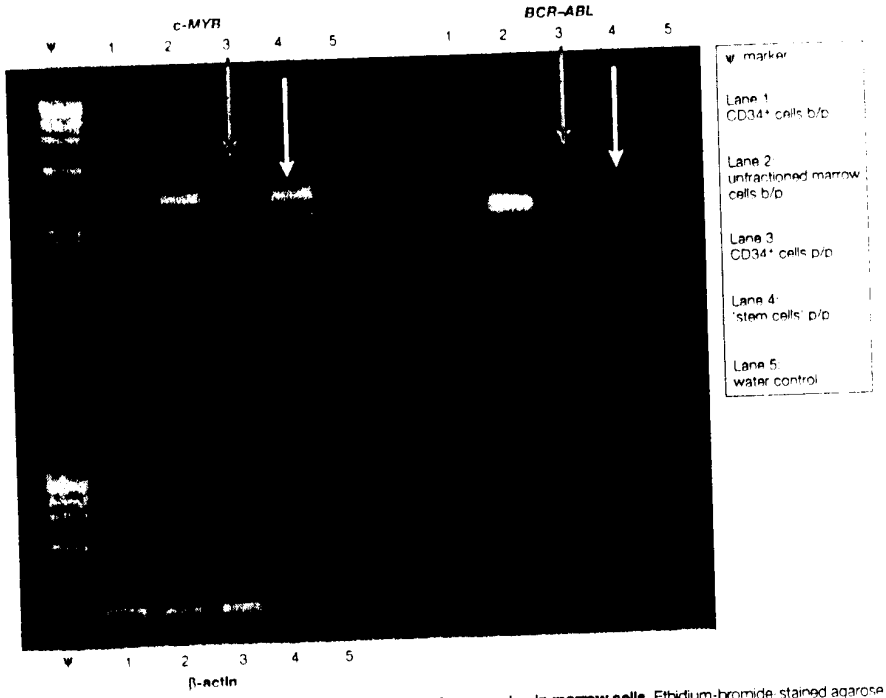

Figure 4 | **Effect of c-*MYB*-targeted ODNs on c-*MYB* mRNA expression in marrow cells.** Ethidium-bromide-stained agarose gel containing c-*MYB*, *BCR–ABL* and β-actin messenger RNA reverse transcriptase (RT)-PCR products derived from CD34+ bone-marrow cells of a representative patient before anti-c-*MYB* oligodeoxynucleotide purging (Lane 1); unfractionated bone-marrow cells before purging (Lane 2); CD34+ cells post-purging (Lane 3); and the patient's primitive 'stem cells' post purging (Lane 4). A control RT-PCR reaction that contains only water is shown in Lane 5. Lanes containing molecular-weight markers are indicated by the symbol M. Lane 3 (orange arrows) reveals that c-*MYB* mRNA is undetectable post purging, whereas some residual *BCR–ABL* expression (molecular marker of the malignant cells) persists. Efficiency of the process on primitive haematopoietic cells is shown in lane 4 (white arrows). Here, stem cells, cultured for ten days post-purge, show normal MYB expression, whereas *BCR–ABL* expression is undetectable. These data indicate that, in this patient's marrow sample, normal cells survived the purge but malignant, *BCR–ABL*-expressing cells did not. Control cells that were treated in an identical manner but not exposed to the anti-c-*MYB* oligodeoxynucleotide continue to express *BCR–ABL* (not shown), which indicates that the results are due to oligodeoxynucleotide exposure and are not a cell-culture artefact. b/p, before purging; p/p, post purging.

out of fourteen patients had originally obtained a 'major' cytogenetic response. Conclusions about clinical efficacy of ODN marrow purging could not be drawn from this small pilot study. Nevertheless, these results led the authors to speculate that enhanced delivery of ODN, targeted to crucial proteins with short half-lives, might lead to the development of more effective nucleic-acid drugs and enhanced clinical utility of these compounds in the future.

Oncogenic signal-transduction pathways

Protein kinase C-α. Protein kinase C (PKC) comprises a family of biochemically and functionally distinct phospholipid-dependent, cytoplasmic serine/threonine kinases. These proteins have a crucial role in transducing the signals that regulate cell proliferation and differentiation. PKC is overexpressed in several tumours, and antisense inhibitors of these enzymes have shown some antitumour activity *in vitro*[82,94] and in animal models[95]. Results of two studies that used the identical 20-mer phosphorothioate ODN against *PKCα* have been published[82,85]. The ODN was well tolerated, but antitumour effects were modest at best. Correlations with levels of *PKCα* expression were not provided.

RAS pathway

h-RAS oligonucleotide. h-RAS is a powerful regulator of several interconnected receptor-signalling pathways. The gene is constitutively active, and promotes proliferation and malignant transformation in many human tumours. Cunningham *et al.* reported results from a study that was carried out on 23 patients with various malignancies[96]. As in other studies with phosphorothioate oligonucleotides, only mild toxicities were observed. No complete or partial responses were achieved. Four patients had stabilized disease for 6–10 cycles of treatment.

c-RAF kinase. RAF proteins are crucial effectors in the RAS signal-transduction pathway. Constitutive activation of the RAS pathway is thought to contribute to malignant transformation in many cell types, which makes elements of this signalling pathway attractive targets for inhibition. Effectiveness of an antisense oligonucleotide against *c-RAF* has been shown both *in vitro*[97] and in an *in vivo* tumour-xenograft model[98]. On the basis of this work, three clinical trials were initiated[99,119,148]. A total of 78 patients were treated. No major tumour responses were documented, but some patients had stabilization of their disease.

Ribozymes

Ribozymes have been the subject of several authoritative reviews[41,100]. Although there is a comprehensive literature that describes the use of these molecules to target a wide variety of mRNA species in various cell-free, cell-intact and animal-model systems (see REFS 41,111), there is little recently published material on the use of these materials in clinical trials. The earliest clinical use of ribozymes was in patients with HIV[77,78,101,102]. As is true of antisense oligodeoxynucleotides, the approach was found to be safe when ribozymes were expressed in cells that were then delivered back to patients, but clinical efficacy was found wanting. At present, several Phase I/II clinical trials with exogenously delivered synthetic ribozymes are in early-phase clinical evaluation for patients with breast cancer, colon cancer and hepatitis. Results of these clinical investigations are anxiously awaited.

Studies in non-malignant diseases

Inflammatory diseases. Antisense oligonucleotides have been explored as anti-inflammatory agents. An example is the targeting of intracellular adhesion molecule-1 (ICAM-1) in Crohn's disease. In response to inflammatory stimuli, many cells upregulate the expression of *ICAM1*, which has an important role in the transport and activation of leukocytes. It has been shown *in vitro* and *in vivo* that administration of antisense oligonucleotides against *ICAM1* causes a decrease in receptor expression, which in turn ameliorates inflammatory reactions[103–105]. Two clinical trials with this compound in patients with Crohn's disease have been reported[6,106]. In the double-blind study reported by Yacyshyn *et al.*[6], 20 patients were randomized to receive a saline placebo or anti-*ICAM1* antisense oligonucleotide. The treatment was well tolerated, and after 6 months, disease remission was reported in 47% of patients in the antisense group compared with 20% of patients in the placebo group. Furthermore, corticosteroid usage was significantly lower ($p = 0.0001$) in the antisense-treated patients. These results engendered a great deal of excitement, but the enthusiasm was subsequently dampened by the follow-on study that was carried out with this compound in a larger group of patients with this disease ($n = 75$)[106]. In this placebo-controlled study, no statistically significant differences in steroid use between the treatment or placebo groups was observed, although 'positive trends' were seen in the patients who were treated with the antisense oligonucleotide. As with other studies, toxicity was mild and consisted primarily of pain at the injection site, fever and headache.

The anti-*ICAM1* oligonucleotide has also been evaluated in patients with psoriasis. The drug was initially administered by intravenous infusion to these individuals, but examination of their skin indicated that delivery to its various layers was poor. For this reason, a topical formulation was developed. Although preclinical data about uptake of this formulation into the skin and downregulation of expression of the target were encouraging[107], the ensuing clinical trial showed only modest, short-term effects in these patients (see the ISIS Pharmaceuticals web site online). The ultimate usefulness of this compound remains to be determined.

Cardiovascular disease. RESTENOSIS of coronary vessels after *trans*-catheter re-vascularization procedures remains a serious clinical problem. Manipulation of coronary vessels invariably leads to endothelial-cell injury, which is often accompanied by thrombosis, smooth-muscle-cell activation and subsequent vascular remodelling. The myelocytomatosis viral oncogene homologue (c-MYC) has been identified as an important mediator in this process through its effects on regulating the growth of vascular cells in atherosclerotic lesions. Accordingly, it has been postulated that *c-MYC* might make an attractive target for preventing post-angioplasty complications, and at least two clinical trials using a 15-mer phosphorothioate-modified antisense ODN against c-*MYC* have been reported[108,109]. Both studies showed safety of intracoronary application of the drug, but no objective clinical responses.

Oligonucleotides as immunological adjuvants

Over the past several years, it has become increasingly appreciated that several types of immune cell have pattern-recognition receptors that can distinguish prokaryotic DNA from vertebrate DNA[110]. This is apparently accomplished by the ability of these receptors to recognize unmethylated CpG dinucleotides in certain base contexts (CpG motifs)[111]. Bacterial DNA, or more germane to this discussion, synthetic oligodeoxynucleotides that contain these unmethylated CpG motifs, can activate immune responses that have evolved to protect the host against infections. Responses of this type are similar to T-helper type 1 (T_H1)-cell responses, and lead to activation of natural killer (NK) cells, dendritic cells, macrophages and B cells[112]. CpG DNA-induced immune activation has been shown to protect certain hosts against infection, either alone, or in combination with vaccines. It is reasonable to suppose, then, that CpG containing oligonucleotides might prove to be effective adjuvants for the immunotherapy of cancer, and for boosting immune responses to antigens that are less efficient in this regard, but to which one would like to immunize a host[113].

The most recent application of this principle was reported in abstract form at the December 2001 meeting of the American Society of Hematology, where preliminary results from a clinical trial in which the

RESTENOSIS
A reduction in luminal size after an inter-arterial coronary intervention.

Table 2 | **Current and planned clinical trials with antisense oligonucleotides and ribozymes**

Product	Diseases	Company
Anti-c-MYC (AS)	Cardiovascular restenosis, Phase II	[illegible]
EPI 2010 (AS against adenosine A1 receptor)	Asthma, Phase II	[illegible]
Genasense (AS against BCL2)	Haematological malignancies Solid tumours, Phase III	[illegible]
GTI 2040 (AS against ribonucleotide reductase)	Solid tumours, Phase I and II	[illegible]
HGTV (AS against HIV)	HIV, Phase II	[illegible]
CpG molecules	Solid tumors Infectious diseases, Phase I/II	[illegible]
Angiozyme (Ribozyme against VEGFR1)	Breast and colon cancer, Phase II	[illegible]
Heptazyme (Ribozyme against HCV)	HCV, Phase I	
Herzyme (Ribozyme against HER2)	Breast and [illegible], Phase I	
ISIS 3521 (PKC-α)	NSCLC, NHL, Phase III	ISIS Pharmaceuticals
ISIS 5132 (c-RAF)	Solid tumours, Phase II	
ISIS 2503 (h-RAS)	NSCLC, Phase II	
G 3139 (BCL2)	NHL, Phase II/III	
GEM 231 (PKA)	PKA, Phase II	

AS, antisense; BCL2, B-cell lymphoma protein 2; CpG, unmethylated CpG dinucleotides; HCV, hepatitis C virus; HER2, tyrosine-kinase growth-factor receptor, also called c-ERBB2; HIV, human immunodeficiency virus; c-MYC, myelocytomatosis viral oncogene homologue; NHL, Non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PKA, protein kinase A; PKC-α, protein kinase C-α; VEGFR1, vascular-endothelial-growth-factor receptor 1.

safety and efficacy of a CpG adjuvant was investigated in 16 patients with non-Hodgkin's lymphoma were reported[114]. Analysis of the data accrued at the time of submission indicated that the oligonucleotide increased the number and activity of NK cells in treated patients, and 2 out of 16 treated patients achieved partial remission. The study is continuing, and a follow-on trial of the CpG oligonucleotide in combination with rituximab is being planned.

Problems in need of solution

Nucleic-acid-mediated gene silencing has been used with great success in the laboratory[107,115–117], and this strategy has also generated some encouraging results in the clinic[90,93,96,118,119]. Nevertheless, it is widely appreciated that the ability of nucleic-acid molecules to modify gene expression *in vivo* is quite variable, and therefore wanting in terms of reliability[120,121]. Several issues have been implicated as a root cause of this problem, including molecule delivery to targeted cells and specific compartments within cells, and identification of sequence that is accessible to hybridization in the genomic DNA or RNA[2]. Intuitively, DNA accessibility is limited by compaction of nuclear material and transcription activity of the gene target. Formal approaches for solving this problem have not been widely discussed. In mRNA, sequence accessibility is dictated by internal base pairing and the proteins that associate with the RNA in a living cell. Attempts to accurately predict the *in vivo* structure of RNA have been fraught with difficulty[122]. Accordingly, mRNA targeting is largely a random process, which accounts for the many experiments in which the addition of an antisense nucleic acid yields no effect on expression. Several approaches to this problem have been tried, including trial-and-error 'walks' down the mRNA[123], computer-assisted modelling of RNA structure[124], hybridization of RNA to random oligonucleotides arrayed on glass slides[125,126] and variations on the theme of using random oligonucleotide libraries to identify RNase H cleavable sites, in the absence or presence of crude cellular extracts[127,128]. Recent work from this laboratory indicates that self-quenching reporter molecules might be useful for solving *in vivo* RNA structure[129], but the reliability and usefulness of this approach remain to be proven.

Another problem in this field is the limited ability to deliver nucleic acids into cells and have them reach their target[129]. Without this ability, it is clear that even an appropriately targeted sequence is not likely to be efficient. As a general rule, oligonucleotides are taken up primarily through a combination of adsorptive and fluid-phase endocytosis[130–133]. After internalization, confocal and electron microscopy studies have indicated that the bulk of the oligonucleotides enter the endosome–lysosome compartment, in which most of the material becomes either trapped or degraded. Biological inactivity is the predictable consequence of these events. Nevertheless, oligonucleotides can escape from the vesicles intact, enter the cytoplasm and then diffuse into the nucleus, where they presumably acquire their mRNA, or in the case of decoys, protein target[133,134]. Delivery technologies continue to improve, so it is likely that present methods, and/or other evolving technologies, will be used successfully to deliver optimized nucleic acids to their cellular targets[135,136]. Indeed, it is our hypothesis that development of

effectively targeted and efficiently delivered nucleic-acid molecules will lead to important advances in the diagnosis and treatment of human malignancies[91], and other diseases for which this class of molecule has been proposed to be effective.

In addition to delivering and targeting oligonucleotides to the mRNA, we believe that other considerations might improve the efficacy of this strategy. In this regard, we suggest that the abundance and half-life of the target mRNA should also be considered when selecting a gene target. The *c-MYB* mRNA that we have chosen to target, as well as its encoded protein, has an estimated half-life of ~30–50 minutes[137,138]. By contrast, BCL2, for example, has a half-life that has been estimated at ~14 hours[139], and RAF and RAS have half-lives that are estimated to be >24 hours[140,141]. Attempts to eliminate these proteins from cells using oligonucleotides might therefore prove more difficult. Whether these considerations will apply to extremely long lived or endogenously expressed antisense vectors, remains to be seen. As the efficiency of these molecules for perturbing gene expression improves, an important consideration in target selection will be the relative selection in the target versus non-targeted tissue. The ability to target genetic polymorphisms, or cells affected by loss of heterozygosity, might be an effective solution to this problem[142]. Finally, another approach for improving the effectiveness of nucleic-acid drugs as anticancer agents that is under intense investigation is to combine them with more traditional therapeutic modalities. Although this might well prove useful, we strongly believe that it remains important to continue to explore strategies that are designed to promote more reliable and efficient gene silencing with oligonucleotides alone. As discussed above, a prime motivating force for developing these drugs is the hope for non-toxic therapies. Adding back chemotherapy, although perhaps useful in the short term, is in the end counter-productive to this specific goal, unless it can be used at significantly reduced dosages. So far, this has not been the case.

Conclusions

The concept of inhibiting gene expression with antisense nucleic acids developed from studies that were initiated almost a quarter of a century ago[13,14]. Despite the fact that the mechanism by which these molecules modulate gene expression is not always certain[12,128,143], clinical development of antisense compounds has proceeded to the point at which several nucleic-acid drugs have entered Phase I/II, and in a few cases, Phase III trials. Others are about to begin, or are in the late planning stages (TABLE 2). The original motivation for developing these molecules remains strong. The recent development of leukaemia cells that are resistant to the small-molecule inhibitor Gleevec provides another incentive. Although a cell might be able to evolve mutated proteins that evade a small-molecule protein inhibitor, this cannot happen if the mRNA that encodes that protein is no longer made. Accordingly, although only one antisense drug has received FDA approval so far[144], all of the investigators who have laboured long and hard in this field hope that the time to celebrate significant achievements in the clinic will shortly be forthcoming.

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